Invited review: mesenchymal progenitor cells in intramuscular connective tissue development

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(Received 17 March 2015; Accepted 23 July 2015; First published online 9 September 2015)

The abundance and cross-linking of intramuscular connective tissue contributes to the background toughness of meat, and is thus undesirable. Connective tissue is mainly synthesized by intramuscular fibroblasts. Myocytes, adipocytes and fibroblasts are derived from a common pool of progenitor cells during the early embryonic development. It appears that multipotent mesenchymal stem cells first diverge into either myogenic or non-myogenic lineages; non-myogenic mesenchymal progenitors then develop into the stromal-vascular fraction of skeletal muscle wherein adipocytes, fibroblasts and derived mesenchymal progenitors reside. Because non-myogenic mesenchymal progenitors mainly undergo adipogenic or fibrogenic differentiation during muscle development, strengthening progenitor proliferation enhances the potential for both intramuscular adipogenesis and fibrogenesis, leading to the elevation of both marbling and connective tissue content in the resulting meat product. Furthermore, given the bipotent developmental potential of progenitor cells, enhancing their conversion to adipogenesis reduces fibrogenesis, which likely results in the overall improvement of marbling (more intramuscular adipocytes) and tenderness (less connective tissue) of meat. Fibrogenesis is mainly regulated by the transforming growth factor (TGF) \(\beta\) signaling pathway and its regulatory cascade. In addition, extracellular matrix, a part of the intramuscular connective tissue, provides a niche environment for regulating myogenic differentiation of satellite cells and muscle growth. Despite rapid progress, many questions remain in the role of extracellular matrix on muscle development, and factors determining the early differentiation of myogenic, adipogenic and fibrogenic cells, which warrant further studies.

Keywords: fibrogenesis, intramuscular connective tissue, meat, progenitor cells, muscle

Implications
Intramuscular connective tissue contributes to the background toughness of meat, which is mainly synthesized by intramuscular fibroblasts. Recent studies show that adipocytes and fibroblasts are derived from a common pool of mesenchymal progenitor cells during the early embryonic development. Due to the bipotent developmental potential of these progenitor cells, enhancing their conversion to adipogenesis reduces fibrogenesis, which provides an opportunity to improve marbling and tenderness of meat, thus the overall palatability.

Introduction
Meat quality is determined by flavor, tenderness, juiciness, color, nutritional value and others. Tender meat, which contains more intramuscular fat and less connective tissue is demanded by consumers. Meat tenderness is determined by both the myofibrillar effects and the presence and cross-linking of connective tissue. Myofibrillar contribution to toughness can be partially addressed by aging carcasses, which results in the fragmentation of myofibrils primarily due to proteolysis by calpains (Koohmaraie and Geesink, 2006). On the other hand, postmortem aging is ineffective in improving the tenderness of a meat with high collagen content, due to the resistance of collagen to proteolysis. Thus, meat toughness due to connective tissue is called the ‘background toughness’ of meat (Nishimura, 2010). Consistently, the longissimus muscle in beef cattle contains low collagen and is tenderer while beef from limb muscles possesses higher collagen content and is tougher (McCormick, 1999; Dubost et al., 2013a). In addition, the cross-linking of collagen has even greater influence on meat toughness (McCormick, 1994). Because during

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https://doi.org/10.1017/S1751731115001834 Published online by Cambridge University Press
cooking, collagen is gelatinized, which is hampered due to the presence of cross-linking, contributing to the toughness of meat from old animals (Dubost et al., 2013b). The detailed effects of connective tissue structure, collagen cross-linking, and their impacts on meat tenderness have been previously reviewed (Purslow, 2014).

Intramuscular connective tissue is mainly derived from fibroblasts, which are generated through fibrogenesis, a process referring to the generation of fibroblasts and their synthesis of proteins and other components composing the connective tissue. Fibrogenesis is active during the whole life of animals, particularly during the early developmental stage in utero; connective tissues synthesized inside fetal muscle form primordial perimysium and epimysium of muscle bundles at late gestation (Du et al., 2010). In humans, fibrosis refers to a state of excessive deposition of collagen and other extracellular matrix proteins, which is often elicited by a pathological condition and becomes noticeable during the recovery period (Liu and Pravia, 2010). Lysyl oxidase is a rate limiting enzyme catalyzing cross-linking of collagen fibrils (Borg et al., 1985; Huang et al., 2012b). Available studies demonstrated that the content and cross-linking of collagen are frequently correlated to each other, but the turnover of collagen reduces cross-linking (Archile-Contreras et al., 2010), a process increasing tenderness (Hill, 1967; Archile-Contreras et al., 2011; Purslow et al., 2012).

Intramuscular fat is considered part of the intramuscular connective tissue, and intramuscular adipogenesis is inseparable from fibrogenesis due to closely related developmental origins. However, knowledge regarding regulatory mechanisms, or specific and effective manipulations to augment progenitor cell differentiation to a particular lineage, such as adipogenesis, remains poorly defined. The intent of this review is to provide an overview of current knowledge regarding intramuscular collagen deposition and associated marbling development, and discuss possible mechanisms regulating mesenchymal progenitor cell differentiation focusing on fibrogenesis, and their impacts on muscle growth and meat quality.

**Intramuscular connective tissue structure**

**Organization of intramuscular connective tissue**

All connective tissues (cartilage, bone, blood and interstitial tissue) possess three common components: cells, fibers and ground substance. Extracellular matrix tissue refers to a major portion of intramuscular connective tissues surrounding muscle fibers and other cells, which is composed of collagen, elastin, fibronectin, proteoglycans, and other ground substance components (Purslow, 2014). Embedded in extracellular matrix and connective tissue, there are abundant fibroblasts, adipocytes, immune cells, preadipocytes, mesenchymal progenitor cells, and other stromal vascular cells. Connective tissue and associated proteins organize muscle structure, connect muscle fibers to the bone for locomotion, and also mediate muscle growth and development (Sanes, 2003; Jenniskens et al., 2006). The connective tissues surrounding each muscle fiber, termed endomysium, comprised two layers. The inner layer, termed basal lamina, is a 50 to 100 nm thick layer surrounding the sarcolemma, which connects muscle fibers to extracellular niche environment and regulates myogenesis (Wang et al., 2014), and muscle growth (Velleman, 1999). Outside of the endomysium, a thin layer of connective tissue, which integrates into thicker layers between muscle bundles, termed perimysium, and surrounding each muscle, termed epimysium. These connective tissues connect muscle fibers and bundles together, and maintain muscle integrity. Intramuscular adipocytes, blood vessels and nerves are integrated into the connective tissue matrix of the muscle.

**Connective tissue structure**

Collagen is the major component of connective tissue. There are a number of different types of collagen, which are derived from more than 30 genes (Myllyharju and Kivirikko, 2004; Veit et al., 2006; Soderhall et al., 2007). However, in muscle, types I and III collagen are dominant (Light et al., 1985). The ratio of type I to III may be altered depending on muscle types, locations and animal ages (Listrat et al., 1999).

In mature bovine muscles, type I collagen is more abundant in perimysium, but type III collagen levels are enriched in the endomysium (Mayne and Sanderson, 1985). In rats, during aging, the proportion of type I collagen increased, while type III collagen decreased (Kovanen and Suominen, 1989); an increase in type I collagen was also observed in the intramuscular connective tissue of beef cattle at around 6 months of age (Listrat et al., 1999). Up to now, most studies about connective tissue in muscle have been focused on types I and III collagens (Sato et al., 1994; Sato et al., 1997; Duarte et al., 2013).

Each collagen molecule contains three helical polypeptide chains, which are interwined. At both ends, however, non-helical regions termed telopeptide regions are found. Lysyl oxidase is a critical enzyme regulating collagen cross-linking (Siegel and Fu, 1976; Siegel et al., 1976). Lysyl oxidase oxidizes lysine or hydroxylysine in the non-helical portions of collagen molecules to aldehydes, which then react with neighboring collagen molecules to form divalent bonds. Therefore, the presence of lysine and hydroxylysine in the non-helical regions is critical in determining cross-linking development (Robins, 2007). The degree of collagen cross-linking differs in animals of different breeds. In our study with Wagyu and Angus cattle, we found that the collagen content and cross-linking are higher in Wagyu, which correlates with less soluble collagen content (Duarte et al., 2013). We also observed that early nutrition affects collagen content and cross-linking in sheep (Huang et al., 2010). In addition, collagens of different muscle types have various degrees of cross-linking, with the collagen in longissimus muscle having less cross-linking than biceps muscle (Dubost et al., 2013a), correlated with meat tenderness. Collagen cross-linking is a slow process, which increases as animals age, and the high degree of cross-linking is one of the primary reasons for the toughness of meat from old animals. On the other hand, collagens undergo consistent turnover,
albeit slower than other proteins. Because newly synthesized collagens do not contain cross-linking, factors that enhance collagen turnover, reduce cross-linking and improve meat tenderness (Purslow, 2014). Indeed, cross-linking was reduced and soluble collagen content was raised in compensatory growing pigs (Kristensen et al., 2002). Collagen turnover, or remodeling, is regulated by metalloproteinases (Woessner, 1991; Murphy, 2010). The expression of metalloproteinases and their inhibitors, the tissue inhibitors of metalloproteinases, are regulated by a number of factors (Clark et al., 2008), such as inflammation and oxidative stress, which affect cross-linking and meat tenderness (Purslow, 2014).

Development of connective tissue

**Fibrogenic cells and adipocytes share common progenitor cells**

During early skeletal muscle development, mesenchymal stem cells first diverge to either myogenic or non-myogenic lineages. Myogenic progenitors further develop into muscle fibers and satellite cells, whereas non-myogenic progenitor cells develop into the stromal-vascular fraction of mature skeletal muscle in which resides adipocytes, fibroblasts and resident mesenchymal progenitor cells (Du et al., 2013). These non-myogenic progenitors have adipogenic and fibrogenic capacity, as well as osteogenic and chondrogenic potential (Joe et al., 2010; Wosczyna et al., 2012). These cells are mainly located in the stromal-vascular fraction of skeletal muscle and are distinct from satellite cells (Joe et al., 2010; Uezumi et al., 2010, 2011 and 2014). Platelet-derived growth factor receptor α (PDGFRα) is a reliable marker for separating these cells, and CD34+ appears to label the same cell population (Joe et al.; 2010; Uezumi et al.; 2010, 2011 and 2014).

The notion that mesenchymal progenitor cells as the common sources of adipogenic and fibrogenic cells are further proven by the co-expression of PDGFRα with fibrogenic markers (Murphy et al., 2011), or PDGFRβ with adipogenic markers (Yang et al., 2013). Transcription factor 4 (TCF4), also known as transcription factor 7-like 2 (Tcf7l2), was first found to be related with limb development by interacting with Wnt signaling pathway (Cho and Dressler, 1998). Subsequent studies demonstrate TCF4 as a fibrogenic marker (Kardon et al., 2003; Mathew et al., 2011). A portion of TCF4+ fibroblasts also express PDGFRα (Murphy et al., 2011), showing the intrinsic relationship between mesenchymal progenitor cells and TCF4+ fibroblasts. Similarly, in our previous studies, we detected the co-expression of PDGFRα with ZFP423, a marker of adipogenic commitment (Yang et al., 2013). The lack of TCF4+ and ZFP423 co-expressed cells show the divergence of the fibrogenic and adipogenic lineages during progenitor differentiation.

**Mechanisms regulating fibrogenesis**

Transforming growth factor (TGF)-β is the most important profibrogenic cytokine (Liu and Pravia, 2010). TGF superfamily contains several structurally related subfamilies, including TGF-β, bone morphogenetic proteins and activin. Three isoforms of TGF-β have been identified, which are TGF-β1, TGF-β2 and TGF-β3. The TGF-β1 isoform is primarily expressed in endothelial cells, fibroblasts, hematopoietic cells and smooth muscle cells; TGF-β2 mainly exists in epithelial cells and neurons; and TGF-β3 is specifically expressed in mesenchymal cells (Ghosh et al., 2005). All TGF-β isoforms activate downstream SMAD signaling (Attisano and Wrana, 1996; Letterio and Roberts, 1998). The SMAD family contains five receptor-regulated SMAD (R-SMAD 1, 2, 3, 5 and 8), a common SMAD (Co-SMAD 4), and two inhibitor SMAD (I-SMAD 6 and 7) (Moustakas et al., 2001). The ligand, TGF-β, first binds to TGF-β receptor II (TβRII), which then recruits and activates TβRI. Then SMAD2 and SMAD3 are phosphorylated and subsequently bind to SMAD4 (Suwanabo et al., 2011), and the resulting SMAD complex is translocated into the nucleus where it binds to SMAD-specific binding elements of target genes, thereby activating the expression of fibrogenic genes including procollagen and enzymes catalyzing collagen cross-linking (Massague and Chen, 2000). As an anti-inflammatory cytokine, TGF-β signaling is enhanced by inflammation (Bhatnagar et al., 2010; Voloshenyuk et al., 2011), while inhibited by anti-inflammatory factors (Wang et al., 2012).

Connective tissue growth factor (CTGF) is a crucial switch to regulate downstream fibrotic progress (Grotendorst, 1997; Leask et al., 2004). CTGF is a member of CCN family, which are cysteine rich proteins. CTGF gene expression is induced by TGF-β-activated Smad3 binding to its promoter region (Denton and Abraham, 2001; Holmes et al., 2001). Then, CTGF directly stimulates fibroblast proliferation and ECM deposition (Shi-Wen et al., 2008; Morales et al., 2011). Wingless/int (Wnt) signaling pathway plays a crucial role in cell fate commitment (Dorsky et al., 1998; Ross et al., 2000), and synergizes with TGF-β signaling to promote connective tissue synthesis and fibrosis (Brack et al., 2007; Zhou et al., 2012; Cisternas et al., 2014).

Ski/sno family includes ski and sno, which has four distinct isoforms SnoN, SnoN2, SnoA and Sno1 (Nomura et al., 1989; Pearson-White, 1993; Pelzer et al., 1996). Ski/sno family acts as negative regulators of TGF-β pathway by functioning on the downstream signal molecules R-smad/Co-smad complex (Luo, 2004; Deheuninck and Luo, 2009; Jahchan and Luo, 2010), thus reducing connective tissue deposition.

MicroRNAs regulate cell differentiation through inhibiting the expression of target genes. MiR-101a inhibits fibrosis by targeting the TβRI on cardiac fibroblasts (Zhao et al., 2015). High glucose increases the activity of transcriptional co-activator p300, which subsequently enhances the activity of TGFβ pathway by inducing Smad2 acetylation (Bugeyi-Twum et al., 2014). Besides, ERK5, one of the MAPK family members, is a critical regulator in TGF-β1-induced lung fibrosis by enhancing Smad3 acetylation (Kim et al., 2013). A number of cytokines and growth factors, which are involved in the regulation of fibrogenesis are listed in Table 1.
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Table 1 Factors enhancing and decreasing intramuscular fibrogenesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Fibrogenesis</th>
<th>Summary</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ</td>
<td>Up</td>
<td>A key pathway driving fibrogenesis through Smad signaling</td>
<td>Poncelet and Schnaper (2001); Liu and Pravia (2010)</td>
</tr>
<tr>
<td>Inflammatory cytokines</td>
<td>Up</td>
<td>Inflammatory cytokines, such as TNFα, IL-1α, IL-1β and others, promote fibrogenesis through enhancing TGFβ expression</td>
<td>Bhatnagar et al. (2010); Voloshenyuk et al. (2011)</td>
</tr>
<tr>
<td>Wnts</td>
<td>Up</td>
<td>Wnt signaling synergizes with TGFβ signaling to promote fibrogenesis</td>
<td>Zhou et al. (2012); Cisternas et al. (2014)</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Up</td>
<td>Promotes the proliferation of fibroblasts and fibro/adipogenic progenitor cells</td>
<td>Iannaccone et al. (1995); Virag et al. (2007)</td>
</tr>
<tr>
<td>CTGF</td>
<td>Up</td>
<td>Promotes fibroblast proliferation and fibrogenic protein deposition</td>
<td>Shi-Wen et al. (2008); Morales et al. (2011)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Up</td>
<td>Stimulates proliferation of fibroblasts and enhances TGFβ signaling</td>
<td>Zhao et al., (2013); Makihara et al. (2015)</td>
</tr>
<tr>
<td>Anti-inflammatory factors</td>
<td>Down</td>
<td>Anti-inflammatory factors down-regulate TGFβ signaling through inhibiting inflammation</td>
<td>Wang et al. (2012)</td>
</tr>
<tr>
<td>Ski/SnoN</td>
<td>Down</td>
<td>Ski/SnoN family of oncoproteins bind to Smad proteins to inhibit the expression of TGFβ</td>
<td>Liu et al. (2001)</td>
</tr>
<tr>
<td>Zfp423</td>
<td>Down</td>
<td>Zfp423 promotes adipogenic differentiation of adipogenic progenitor cells, which reduce fibrogenesis</td>
<td>Huang et al. (2012a)</td>
</tr>
<tr>
<td>MMPs</td>
<td>Down</td>
<td>Catalyze connective tissue degradation and promote extracellular tissue remodeling</td>
<td>Balcerzak et al. (2001)</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Up</td>
<td>Inhibits MMPs and connective tissue remodeling</td>
<td>Balcerzak et al. (2001)</td>
</tr>
</tbody>
</table>

CTGF = connective tissue growth factor; FGF-2 = basic fibroblast growth factor; MMPs = matrix metalloproteinase; PDGF = platelet-derived growth factor; TGFβ = tumor growth factor β; TIMP = tissue inhibitor of metalloproteinase; Wnts = wingless and ints.

Antagonistic effects of adipogenesis on fibrogenesis

Because fibrogenesis and adipogenesis are considered as a competitive process, enhancing adipogenesis reduces fibrogenesis. Adipogenesis can be separated into two steps, the commitment of progenitors to preadipocytes, and the differentiation of preadipocytes to mature adipocytes. Quite recently, Zfp423 was identified as the key regulator committing progenitors to preadipocytes; in addition, Zfp423 promotes the expression of peroxisome proliferator-activated receptor γ, the crucial transcription factor inducing the conversion of preadipocytes to adipocytes (Gupta et al., 2010; Gupta et al., 2012). Importantly, in cattle mesenchymal progenitor cells, the expression of Zfp423 is negatively correlated with TGF-β1 expression, indicating the mutual exclusion of adipogenesis and fibrogenesis (Huang et al., 2012a).

Connective tissue and muscle development

Satellite cells are critical for muscle growth and regeneration. They are wedged between the basal lamina and the plasma membrane (sarcolemma) of skeletal muscle fibers. Extracellular matrix together with growth factors and cytokines sequestered inside and those secreted by interstitial cells, forms the niche environment needed for satellite cell quiescence, activation, migration, myogenic differentiation and muscle development (Rhoads et al., 2009; Dodson et al., 2010; Murphy et al., 2011; Urciuolo et al., 2013).

Muscle regeneration involves extensive proliferation and myogenic differentiation of satellite cells. Shortly after muscle injury, both satellite cells and non-myogenic progenitor cells are activated and proliferate; non-myogenic progenitor cells stimulate satellite cell proliferation and facilitate muscle regeneration (Joe et al., 2010; Murphy et al., 2011). In addition, intramuscular fibroblasts particularly promote slow myogenesis, thus affecting muscle fiber type composition and overall maturation during muscle development (Mathew et al., 2011). Extracellular component, collagen VI, regulates satellite cell self-renewal and differentiation (Urciuolo et al., 2013). Besides, other components of extracellular matrix, such as proteoglycan, regulate proliferation and differentiation of satellite cells (Zhang et al., 2007). Decorin, a small leucine-rich proteoglycan, traps TGFβ to regulate satellite cell activation and muscle growth (Li et al., 2006 and 2008).

Extracellular matrix also interacts with a number of growth factors, including TGFβ, hepatocyte growth factor, fibroblast growth factor 2, myostatin and others to either promote or inhibit muscle growth (Yamaguchi et al., 1990; Rapraeger et al., 1991; Allen et al., 1995; Miura et al., 2006; Kishioka et al., 2008). Table 2 lists selected growth factors known to interact with extracellular matrix and regulate muscle growth.

Conclusions

Intramuscular connective tissue regulates muscle growth and development, and also is the site for intramuscular fat (marbling) deposition. The abundance and cross-linking of

https://doi.org/10.1017/S1751731115001834 Published online by Cambridge University Press
intramuscular connective tissue contribute to the background toughness of meat. Connective tissue is mainly synthesized by intramuscular fibroblasts. Non-myogenic mesenchymal progenitor cells are the common source of fibroblasts and adipocytes. Strengthening progenitor cell formation and proliferation enhances both intramuscular adipogenesis and fibrogenesis, while enhancing progenitor differentiation to adipogenesis reduces fibrogenesis, resulting in the overall improvement of marbling and tenderness of meat. Fibrogenesis is mainly regulated by the TGF-β signaling pathway, and a number of factors affect connective tissue deposition via altering TGF-β signaling. Extracellular matrix, a part of the intramuscular connective tissue, provides a niche environment to regulate myogenic differentiation of satellite cells and muscle growth. Despite rapid progress in our understanding of mechanisms regulating fibrogenesis, many questions remain on the synthesis of intramuscular connective tissue and the role of extracellular matrix in muscle development, which warrants further studies.

Acknowledgements

This project was supported by Agriculture and Food Research Initiative Competitive Grant No. 2015-67015-23219 from the USDA National Institute of Food and Agriculture, and NIH R01 HD067449.

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